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Aus der Abteilung für experimentelle Medizin der
F. Hoffmann-La Roche & Co., AG., Basel
Arbeit unter Leitung von PD. Dr. med. A. Studer

The Effect of Partial Hepatectomy upon the Rate of Mitoses in Carbon Tetrachloride Induced Liver Damage of Parabiotic Rats

Inaugural-Dissertation
zur Erlangung der Doktorwürde
der Medizinischen Fakultät der Universität Basel

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Introduction

Regeneration of the tissue, especially glandular tissue, is a well-established biological phenomenon. As early as 1896 *Podwyssozki* (1) described the process in liver; its histological proof was later provided by *Ponfick* (2), and in 1894 by *von Meister* (3). *Podwyssozki* (1) removed bits of liver tissue and noted that the animal soon restored the original liver weight to body weight ratio – this phenomenon he called regeneration. *Ponfick* (4) showed that this process was accompanied by the appearance of an increase in mitotic figures. He claimed that in normal liver tissue one never (or rarely) finds mitoses. At the site of the operation, however, there was evidence of increasing mitoses, especially what he described as "Gallengang-Wucherung" (i.e. growth by bile capillaries and these cells becoming liver cells). This idea of growth, or regeneration, although shown to be otherwise by *Cameron* and *Karunaratne* (5), is still believed by some. Further, the studies of *Brues* et al. (6) have shown that the mitotic figures are equally spread throughout the liver tissue and show no preference for either the site of the operation or for the bile capillaries. *Brues* and *Marble* (7) found the normal mitotic rate in the white rat to be 1 in every 10,000–20,000 normal cells.

Interest in this regeneration was further stimulated when studies showed that the liver could, after having been reduced to 30% of its former size by partial hepatectomy, regain its former size in a very few days, and have reconstituted its histological structure in about two weeks time. The most rapid segment of this growth took place within the first 48–72 hours. The study of this regeneration interested both biochemists and histologists (8, 9, 10, 11, 12, 13, 14, 15, 16). It was through the work of these and many others that explanations of this phenomenon came to being. For example, *Higgins* and *Anderson* (8) attempted to explain it as a direct correlation to the blood flowing through the remaining liver, i.e. via mechanical stimulus. Since the remaining liver tissue received all the blood that previously flowed through the entire liver, and since it has been shown that regeneration after partial hepatectomy can be decreased, indeed prevented, by an Eck fistula (17) or by ligation of the portal vein (18); – or conversely, regeneration can be increased in tempo by increasing the blood flow through the portal system via anastomosis (19), it followed that regeneration was in some way dependent on the blood flow. It was therefore postulated that the distention of the sinusoids resulted in hypertrophy of the cells and this, plus mitosis, explained all that was involved in the hepatic restoration.

If one were to assume that the above theory was the explanation of liver regeneration, it would be rather difficult to explain the results that *Bucher*

et al. (20) claim for their animals. The latter have shown that in parabiotic twins and triplets a process identifiable histologically as regeneration occurs in the undamaged twin when the other twin has been partially hepatectomized. Since the only biological connection between the two animals is that of a crossed-blood circulation, and since the increase in the flow of blood to the undamaged partner's liver, if any, can be considered inconsequential, *Bucher* et al. (20) came to the conclusion that the activation of this process must be blood borne, – therefore a biochemical, not a mechanical factor. *Glinos* (21) discussed the possibility of a hormonal influence and, with others, sought it in the serum proteins. Recently regeneration was shown in the fatty liver after partial hepatectomy (16).

It has been shown, therefore, that when two normal animals are united parabiotically, the regenerating liver, after partial hepatectomy of the one partner can definitely influence the mitotic rate in the liver of the other.

This paper presents the following problems: 1. to repeat the work of *Bucher* et al. (20), as this is the basis of the proposition that the mitotic impulse can be transmitted from the partially hepatectomized rat to the healthy liver of its parabiotic partner, and 2. to attempt to transmit this impulse to the liver of the non-hepatectomized parabiotic partner that was previously treated with carbon tetrachloride so as to induce liver damage.

Materials and Methods

In our experiments white Wistar rats, all females of 120–150 g, from an inbred strain of our own stock, were used. For parabiosis only sisters of the same litter were paired.

In preliminary experiments single doses of 0.1 cc of carbon tetrachloride (diluted in 0.4 cc of paraffin oil) were found to be too weak. However, 0.125 cc of carbon tetrachloride in 0.375 cc of paraffin oil was found to give enough and more constant damage. Further experiments have shown that the paraffin oil alone, given in repeated doses intraperitoneally, was not capable of producing a mitotic impulse in the normal liver. Also in the early experiments with repeated intraperitoneal injections of carbon tetrachloride – paraffin oil mixture there was no mitotic impulse in the damaged liver to be found.

Colchicine. The mitotic activity is measured by means of the *Dustin* (22) reaction which proved to be good also in *Studer's* (23) experiments. By intraperitoneal injection of Colchicine a "blockade" of the mitoses in the metastasis is obtained. As previous experiments have shown, 1 γ/g of body weight will noticeably increase the number of mitoses of liver cells that can be perceived, without, however, increasing the small mitotic rate of a normal liver to any noticeable degree. Because of the toxicity of Colchicine and because the animals were weakened by the experiments, some died before the end of the experiment. In our results, however, only those animals that lived

at least 6 hours after the injection of Colchicine were considered. Those that lived beyond this minimum were sacrificed at 9½ hours after the injection.

Liver damage. Methods to experimentally damage the liver of laboratory animals have long been in the hands of the experimenter. The studies of liver damage via diet (11, 24, 25, 26, 27, 28, 29, 30) are well known, further, damage can be induced by bile duct obstruction (15), by drugs (31), metals (32), allyl alcohol (33), and even water (34). Due to the wealth of material, references, and reference standards it was found to be very convenient to use carbon tetrachloride as our liver damaging substance.

Carbon tetrachloride was first brought into the hands of the experimenter in connection with its therapeutic use as an antihelminth. It was found that liver damage was caused by overdoses and it was *Gardner* (35) that did much to describe the histological changes in the liver. *Lacquet* (9) in 1932 added the idea of partial hepatectomy to the carbon tetrachloride problem; it is interesting to note that he performed the partial hepatectomy first and then "poisoned" the animals with carbon tetrachloride. He showed a resistance of the regenerating liver to the "poison". The work of *Cameron* and *Karunaratne* (5) in 1936 remains the classical yardstick for dosages and time intervals. However, some investigators have had some difficulty in duplicating the results of *Cameron* and *Karunaratne*. *McCloskey* and *McGehee* (36) believe that the method of administration of the drug is the prime factor. They concluded that oral administration was the method of choice, however, intraperitoneal administration was not included in their work. An attempt was also made here to duplicate the results of *Cameron* and *Karunaratne*.

Parabiosis. The procedure as carried out here is that method described by *Bunster* and *Meyer* (37) with very slight modifications. This method was chosen over that used and described by *Sauerbruch* and *Heyde* (38) (originally from *Bert* [39]), as the latter, the "open coelio-anastomosis", presents the possibility of herniation and intestinal strangulation. The operation proceeds as follows: the animals, previously paired female albino rats of the same litter and of 120–150 g in weight were shaved with an electric razor and then anaesthetized with ether. The animals were then placed on their backs and an incision was made in the skin from the base of the ear to the iliac crest. The ventral skin flaps of both animals were then sutured together with a continuous suture. As a matter of convenience, suturing was started caudally, so that at the very end one would again be at the tail region where there is more room to work. In the second step the animals were turned over and the scapulae exposed. This was accomplished by holding the humerus by its anatomical neck and pushing directly dorsally until the margo vertebralis scapulae is prominent. Holes were then bored into the scapulae with a dental bore. After this was accomplished a strong thread was threaded through the holes and tied. In the third step the abdominal muscles were incised along the mid-axillary line. Care must be taken to avoid damaging the diaphragm and/or the intestinal contents. The incision was, therefore, made well below

the costal margin and extended almost to the iliac crest. The two abdominal incisions were closed and united with the same "through and through" continuous suture – this is the so-called "bridge". Since it has been our experience that soon after (some 8–10 days) the skin of the animals has so "stretched" that the possibility arose that the scapulae union might suffer, some muscle to muscle sutures were placed in strategic places so that the animals had less "play". After this was completed, the dorsal skin flaps were united with the same continuous suture, this time going from the ear to the tail end. To make the recovery easier the animals were bandaged together with Isoplastic.

We now have a physiological unit consisting of two animals with one blood supply. The choice of animals was influenced by the work done by *Bickel* and *Holm-Jensen* (40) and *Van Dyke* et al. (41) and *Huff* et al. (42) who showed that animals from the same litter establish the best "crossed" circulation. *Van Dyke* et al. showed that the establishment of this blood supply takes only 3–4 days. However, since in these experiments both animals were not "normal" at time of operation and since another operative procedure was to be performed, we allowed 10 days for the completion of the "cross" circulation.

Although many of the early workers in the field of parabiosis (43, 44, 45, 46) described parabiotic poisoning and parabiotic death, these were of no consequence in our series. In our earliest experiments, testing the desirability of the different methods of parabiosis, one case of disproportion and death of the smaller animal was seen, but these animals were not chosen and paired correctly.

Hepatectomy (using the method of *Ingle* [48]). Since the animals were now joined parabiotically, it was necessary to anaesthetize both partners – the undamaged partner was shaved with an electric razor from one centimeter above the xiphoid process of the sternum to a line between the two spinae iliacae ventrales. The skin incision was made just below the xiphus sterni and continued along the midline for 2–3 cm. The skin was then prepared free from the underlying tissue as far as possible and retracted. The abdominal incision was then made at right angles to the skin incision at a level just below the lower edge of the liver – that could be easily seen shining through. This incision was about 2 cm. The assistant then took the animal, arched its back, and squeezed gently on the back and lower ribs and lower abdomen until the medial lobe (with its two lobules) and the large left lobe presented themselves. If necessary the operator could pull ever so gently on the presenting liver, until the desired parts were delivered through the incision. It is important to handle the liver as little as possible as it tears at the least provocation and any slight loss of blood by the rat can lead to shock. The delivered lobes were then flapped over the chest wall of the animal and the ligament connecting the medial lobe and the caudal lobe was cut. Both delivered lobes were then ligated at their bases with one and the same liga-

ture. The ligated portion of the liver was then cut and removed. The portion removed was equal to approximately 70% of the total liver tissue (8), and the tiny nobbins trimmed and the liver allowed to fall back into the abdominal cavity. Thrombin (*Topostasin*[1] Roche) was then dusted over the wound to stop all possible bleeding. The abdominal muscle layer was then closed. Then the skin was closed in the usual manner.

Histological preparation and methods of counting the mitoses. On completion of the experiment the animals were sacrificed in ether narcosis. Specimens from one, usually two, different sites in the liver were taken for histological examination and placed in Bouin and formalin respectively; 7 μ sections were cut and stained in the usual way with H. &. E. The counts were made as follows: using 150× (ocular 6×; objective 20×; and tube 1.25×) the mitoses in 30 neighbouring microscopic fields were counted in each of the two specimens. The average of the 60 fields (in group C only 30 fields) represented the mitotic rate of that liver. The decision as to whether a cell was in active mitosis or not depended on the characteristic arrangement of the chromatin and the enlargement of the cell with a homogenous lightening of the stain of the cytoplasm (see fig. 1).

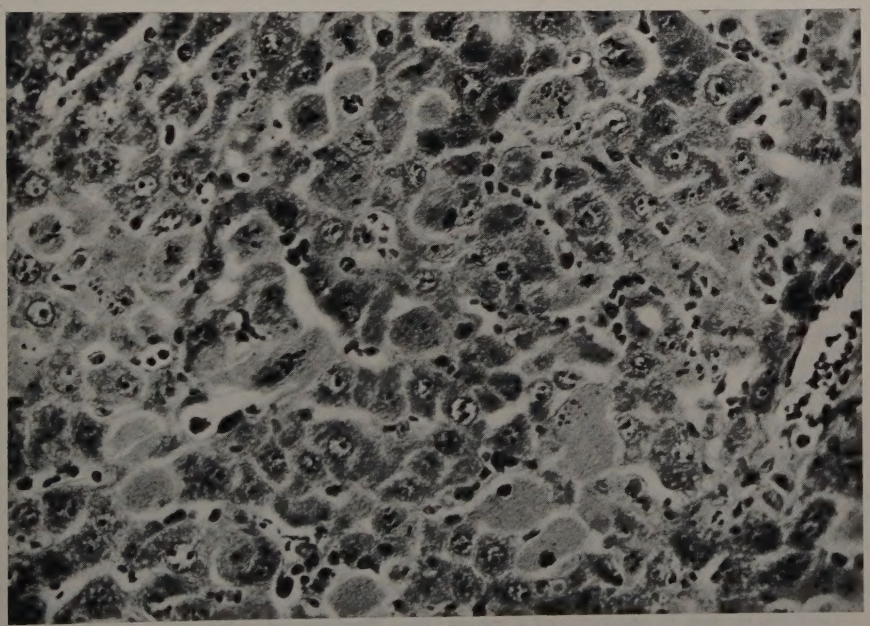

Fig. 1. Rat liver, Hemalaun-Eosin (270×). – 9 hours after colchicine (1 γ/g body weight); 50 hours after partial hepatectomy; 12 days after parabiosis. – One sees many liver cells in mitosis (thickening of the chromatin material and enlargement of the cell).

[1] Trade mark.

Experiments and Results

In *Group A* 13 pairs of animals (sisters of the same litter) were united parabiotically (without preliminary treatment with carbon tetrachloride) and 10 days later one partner was partially hepatectomized. Forty hours later the animals were injected with colchicine, intraperitoneally 1 γ/g of body weight. Five pairs, which only lived 4–5½ hours after the colchicine injection, were not included in the final results. The average mitotic rates (from 30 fields at 150×) for each of the 8 animals with normal livers, which were sacrificed 8–9½ hours after the colchicine injections, are given in Table 1. They show that in every case there is a large increase in mitoses in the partially hepatectomized liver.

The non-hepatectomized partners, however, reacted differently: with rats No. 1, 4 and 5 the increase in mitoses is evident. With rats No. 7 and 8, we have tried to show statistically whether the possibility of a mitotic impulse exists or not (see Discussion). With animals No. 2, 3 and 6 of the same series, there is no mitotic impulse demonstrable.

In *Group B* 20 pairs of animals, selected with the usual prerequisites, were placed in experiment. One member of each pair received an intraperitoneal injection of a mixture of 0.125 cc of carbon tetrachloride and 0.375 cc of liquid paraffin three times each week, until a total of 10 such injections were given. Three days after the last injection the animals were united parabiotically. Ten days after this operation the animal that had not received the carbon tetrachloride was partially hepatectomized; 40 hours later each animal was injected with colchicine 1 γ/g body weight intraperitoneally. In this series all the animals lived over the 6 hour limit and those still alive after 9 jours were sacrificed in the usual manner. However, two pairs could not be considered in the final results. In one case the mitoses could not be attributed with certainty to the liver cells; in the other pair, the partially hepatectomized partner presented an unusual picture, in that its mitotic rate was only about $^1/_{40}$ to $^1/_{10}$ that of the other animals of the series.

Of the 18 pairs that remain of Group B (see Table 1), the partially hepatectomized animals showed the usual increase in mitoses. Of the animals that received carbon tetrachloride in preliminary treatment, we found an increase in mitoses in rats No. 1, 12, and 13. The carbon tetrachloride induced liver damage in these ranged from (+) to +++ (using the system of 0 to +++ to grade the liver damage as estimated by histological examination).

The liver damage, in the stages examined here, consists of an increase of connective tissue and a disappearance of central lobular parenchymal cells.

Control *Group C* consists of 6 pairs; one partner of each pair received the ten intraperitoneal injections of carbon tetrachloride in paraffin oil as described for Group B. Three days after the last injection the animals were united parabiotically and 12 days later, 9½ hours after intraperitoneal injections of colchicine (1 γ/g body weight), the animals were sacrificed.

In the carbon tetrachloride induced liver damaged partner of these six control pairs (which proved to be controls in many ways) there was no demonstrable increase in mitoses. Again the degree of liver damage here ranged from (+) to +++.

Discussion

According to the results of *Bucher* et al. (20), one can demonstrate that an impulse that stimulates mitosis in liver cells can be transmitted from a partially hepatectomized rat to its healthy parabiotic partner. In order to answer our question, i.e. if this impulse, that is elicited by partial hepatectomy, can also be transmitted to a parabiotic partner that has previously had its liver parenchyma damaged with carbon tetrachloride, we must compare groups A and B (Table 1). It is clear, as has long been established, that in the partially hepatectomized liver there is an increase in mitoses. In order to decide if the individual mitotic rates for the untreated partners of group A, as compared with the carbon tetrachloride induced liver damaged partner of group B, were products of our experimental methods or just coincidence, we compiled, under the direction of Mr. *G. Güetli*, the following statistical data and considerations. From the literature (7) and our own preliminary experiments we know that mitoses in the liver of the white rat are very infrequent. This is shown again in group C – the group is, however, too small for statistical considerations. *Brues* and *Marble* (7) found that the mitoses in different equalsized microscopic fields from the same rat followed the Poisson distribution. The Poisson Formula for the possibility of the occurrence of infrequent events states, that the possibility ($P_\varkappa$) of one of the events, repeated n-times (in our case the increase or lack of increase of mitoses) occurring with the other, whose possibility of occurring p is a very small number, is equal to $\varkappa$-times. The other symbols in the following formula are: $\lambda = n \cdot p$ (a positive constant); e = the root of the natural logarithm.

$$\text{Poisson's Formula: } P_\varkappa = \frac{\lambda^\varkappa e^{-\lambda}}{\varkappa!} \quad (\varkappa = 0,1,2,\ldots)$$

If we were to assume that the number of mitoses in different pairs of animals follow the Poisson distribution, then the larger mitotic counts (for example, greater than 9) would be very infrequent. The possibility of a mitotic count equal to, or larger than 10, would, using the example of *Brues* and *Marble*, appear in 1 in 10^6 chances. This means that by counting the mitoses in one million microscopic fields one would find, on the average, only 1 field with 10 or more mitoses.

With the help of this knowledge we can now examine our results of group A and B. Of the 8 undamaged partners of group A, 3 showed mitotic rates of less than 10 and can, therefore, be considered normal. Consequently 5 animals definitely show an increase in mitoses. In Group B, by the same consid-

Table 1

Group A Parabiosis + partial hepatectomy + colchicine			Group B Carbon tetrachloride + parabiosis + partial hepatectomy + colchicine				Group C Carbon tetrachloride + parabiosis + colchicine		
	Mitotic rate			Mitotic rate			Carbon tetrachloride induced damage		
Pair No.	partially hepatectomized partner	undamaged partner	Pair No.	partially hepatectomized partner	carbon tetrachloride induced liver damaged partner	Degree of liver damage (0 to +++)	Rat No.	Mitotic rate	degree of liver damage (0 to +++)
1	749	211	1	295	47	(+)	1	1	(+)
2	882	9	2	876	3	++	2	0	++
3	1230	4	3	684	4	++	3	0	++
4	706	211	4	391	0	+	4	0	++
5	451	519	5	330	1	+	5	0	+(+)
6	890	1	6	769	0	+	6	0	+++
7	128	55	7	636	1	+(+)			
8	638	91	8	443	2	(+)			
			9	318	3	+(+)			
			10	924	1	++(+)			
			11	693	0	+(+)			
			12	1337	15	+			
			13	1054	109	++			
			14	209	5	+			
			15	515	1	+(+)			
			16	1180	0	+++			
			17	668	1	++			
			18	266	1	+(+)			

erations, only 3 of the 18 liver damaged partners show an increase in mitotic rate – in other words, of the 18 partners with preliminary carbon tetrachloride induced liver damage only 3 animals showed increases in mitotic rates as opposed to 5 of the 8 undamaged partners of Group A.

There remains then the statistical proof as to whether the frequency of the increased mitoses of the undamaged partner (Group A) is statistically greater than the frequency of increased mitoses in the carbon tetrachloride induced liver damaged partner (Group B). The results are as follows:

Table 2

The comparison of the frequency of increased mitoses in the liver of the undamaged, and the carbon tetrachloride induced liver damaged partners, respectively, Groups A and B

	Number of animals			Relative frequency
	with increased mitoses	without increased mitoses		
Group A	$5 = k_1$ (62%)	$3 = l_1$	$8 = n_1 = k_1 + l_1$	$h_1 = \frac{k_1}{n_1}$
Group B	$3 = k_2$ (17%)	$15 = l_2$	$18 = n_2 = k_2 + l_2$	$h_2 = \frac{k_2}{n_2}$
	$8 = k_1 + k_2 = K$	$18 = l_1 + l_2 = L$	$26 = n_1 + n_2 = N$	

Testing the results of Table 2 with the Chi-square we have:

$$\chi^2 = \frac{(h_1 - h_2)^2 \cdot n_1 \cdot n_2 \cdot (N-1)}{K \cdot L} = 5.244$$

According to the Tables $P = 0.05$ i.e. $\chi^2_{0,05} = 3.841$. The value found, 5.244 is therefore significant ($P < 0.05$). This significance is also confirmed by the (more) exact method of *Fisher* (47) – as follows:

	positive	negative	combined	Extremes							
				①		②			③		
A	$5 = k_1$	$3 = l_1$	$8 = n_1$	6	2	8	7	1	8	8	0	8
B	$3 = k_2$	$15 = l_2$	$18 = n_2$	2	16	18	1	17	18	0	18	18
	$8 = k_1 + k_2 = K$	$18 = L$	$26 = N$	8	18	26	8	18	26	8	18	26

$$P_k(k_1, k_2) = \frac{n_1! n_2! K! L!}{k_1! l_1! k_2! l_2! N!}$$

$$P = \sum_{k_2 = 3,2,1,0} P_k(k_1, k_2)$$

$$P = \frac{8!18!}{26!} \cdot \left(\frac{8! \cdot 18!}{5! \cdot 3! \cdot 3! \cdot 15!} + \frac{8! \cdot 18!}{6! \cdot 2! \cdot 2! \cdot 16!} + \frac{8! \cdot 18!}{7! \cdot 17!} + \frac{8! \cdot 18!}{8! \cdot 18!} \right) = 0{,}032 \ (< 0{,}05)$$

It is apparent from the above calculations that the mitotic impulse, which is initiated from the partially hepatectomized partner, results in a significantly more frequent increase in mitoses in the undamaged partner than in the partner with carbon tetrachloride induced liver damage.

That the mitotic impulse is due to the partial hepatectomy and is transmitted to the parabiotic partner, is proven by the results of the control group (C), in which there was no partial hepatectomy. From this group, in which there was no increase in mitoses, we can eliminate as possible causes of the impulse 1. the parabiotic operation and its post-operative results, 2. the carbon tetrachloride induced liver damage, and 3. colchicine.

Why three of the eight undamaged animals did not respond to the mitotic impulse – i.e. is it a question of individual resistance or was the mitotic impulse not transmitted because of incomplete parabiotic union – are questions we cannot answer.

If we have established that the mitotic impulse is less frequently transmitted to a parabiotic partner with previous carbon tetrachloride induced liver damage than to an undamaged partner, it would seem logical to assume that the animals with the most (histologically assessable) damage from the carbon tetrachloride, would respond least to the mitotic impulse. First, one must say, that the number of animals used here is too small to evaluate any real correlation. However, it is worthy of note, that in pair No. 8 (Group B) there was no mitotic increase in spite of the insignificant liver damage and, on the other hand, in pair No. 13 (Group B) there is a definite increase in the mitoses despite a definite liver damage. One can therefore assume that there is some "blockade" of the mitotic impulse that is not discernible histologically.

Summary

Using paired, parabiotically united white rats, we compared the results of the effect of the mitotic impulse, induced by partial hepatectomy, on the healthy partner and the partner that had previously received carbon tetrachloride to induce liver damage.

The mitotic impulse, which is elicited from the partially hepatectomized partner generally causes an increase in the mitoses of the liver cells in the undamaged partner (62% of our animals), however, significantly less (17%) in the partner that had previous carbon tetrachloride induced liver damage. The ability of the damaged liver to react to the mitotic impulse is therefore diminished.

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